



NCL Method STE-2.1

Detection of Microbial Contamination

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This protocol assumes an intermediate level of scientific competency with regard to techniques, instrumentation, and safety procedures. Rudimentary assay details have been omitted for the sake of brevity.

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1. Introduction

This protocol describes a procedure for quantitative determination of microbial contamination in a nanoparticle preparation. The protocol includes tests for yeast, mold and bacteria using Millipore Sampler devices [1].

Note: This assay is not intended to certify the material as sterile. The intended purpose is to avoid introduction of microbial contamination into in vitro cell cultures and in vivo animal studies utilizing the test-nanomaterial, as microbial contamination will confound the results of these tests.

2. Reagents and Equipment

Note: The NCL does not endorse any of the suppliers listed below; their inclusion is for informational purposes only. Equivalent supplies from alternate vendors can be substituted.

2.1 Reagents

1. Sterile PBS (GE Life Sciences, SH 30256.01)
2. Yeast and mold sampler (Millipore Corp., MY0010025)
3. HPC Count sampler (Millipore Corp., MHPC10025)
4. Bacterial cell culture for positive control (ATCC, 25254)
5. Yeast cell culture for positive control (ATCC, MYA 774)
6. Sodium Hydroxide (NaOH) (Sigma, S2770)
7. Hydrochloric acid (HCl) (Sigma, H9892)
8. Test nanomaterial
9. Buffer used to reconstitute test nanomaterial

2.2 Materials

1. Pipettes, 0.05 to 10 mL
2. Sterile pipets, 1-10 mL
3. Sterile tubes, 5 mL

2.3 Equipment

1. Incubator, 37°C
2. Vortex

3. Reagent Preparation

3.1 Sodium Hydroxide

Prepare from concentrated stock by dilution into sterile water to make a 0.1 N final concentration solution.

3.2 Hydrochloric Acid

Prepare from concentrated stock by dilution into sterile water to make a 0.1 N final concentration solution.

4. Preparation of Controls

4.1 Negative control (NC)

Use sterile PBS or water as a negative control. The negative control is acceptable if no colony forming units (CFU) are observed upon completion of the test.

4.2 Positive Control (PC)

For the positive control, use bacterial or yeast cell cultures (e.g. ATCC #25254 and MYA774, respectively) at a dilution which will allow at least 10 CFU/mL. If standard cultures are not available, a sample from another source (e.g., rain water, floor swipe, etc.) known to contain bacteria and yeast/mold may be used.

4.3 Inhibition Control

To assess whether nanoparticles inhibit bacterial growth, a positive control sample at the same final dilution as described in section 4.2 is spiked into the test nanoparticle sample. For example, spike 22 CFU per 2.2 mL of nanoparticle solution at a given dilution and use 1 mL for seeding into a paddle as described in section 6 below. The final inhibition control will contain the same concentration of nanoparticles as nanoparticle unspiked sample and the same concentration of bacteria as in the positive control (10 CFU/mL).

5. Study Samples

The assay requires 5 mL of the test nanoparticle. The concentration of nanoparticles is case-specific. Most samples are tested at the supplied stock concentration, and at several serial 1:10 dilutions, i.e. no dilution, 1:10, 1:100, 1:1000. When such information is not available, for example when a test nanomaterial is received from a commercial supplier in a form not intended

for biomedical applications, prepare a solution at 1 mg/mL. The weight information can refer to active pharmaceutical ingredient, total construct, total metal content, or other units. Such information is specific to each nanoparticle and should be recorded to aid result interpretation.

Test nanoparticles should be reconstituted in sterile PBS, water or other appropriate vehicle. If vehicle is a buffer or media other than water or PBS, the vehicle control should also be included in the test. The pH of the study sample should be checked using a pH microelectrode and adjusted with either sterile NaOH or HCl as necessary to be within the pH range 6-8. If NaOH or HCl are not compatible with a given nanoparticle formulation, adjust pH using a procedure recommended by nanomaterial manufacturer. To avoid sample contamination from microelectrode, always remove a small aliquot of the sample for use in measuring the pH.

6. Experimental Procedure

1. Remove the Sampler from its plastic bag and write the date and sample reference number on the case with indelible marker.
2. Under sterile conditions, remove a paddle from the case and apply 1 mL of nanoparticle preparation (or dilution) onto the surface of the filter. Allow liquid to absorb, then recap the paddle. To prevent the paddle from drying out during incubation, it should be seated firmly in the case to form an air-tight seal. Prepare 2 paddles per each sample.
3. Incubate for 72 hr at a nominal temperature of 37°C.
4. Remove paddle from case and examine for appearance of colonies. Count colonies.
5. Report results according to the following formula:

$$\# \text{ Colonies} \times \text{Dilution Factor} = \text{CFU/mL}$$

7 Interpretation of Results and Acceptance Criteria

1. A positive control is considered acceptable if it allows identification of at least 10 CFU/mL.
2. A negative control is acceptable if no colony is detected.
3. A test sample is considered negative if no colony is detected.
4. An inhibition control is considered acceptable if it shows no significant (≥ 2 fold) difference in CFU number from that observed in the positive control.

5. A ≥ 2 fold decrease in the number of colonies in the inhibition control sample versus the positive control sample suggests the nanomaterial has the potential to inhibit bacterial growth. Further investigation, including analysis of minimal inhibitory concentration (MIC), is needed to verify such findings.

8. References

1. Samplers, Dilution kits and swab test kits user guide. P15325, Rev. D., 8/99 Millipore Corp.

9. Abbreviations

CFU	colony forming units
HCl	hydrochloric acid
NaOH	sodium hydroxide
PBS	phosphate buffered saline
MIC	minimal inhibitory concentration